Highly Optimized Flow Vesiculometry™ for Vascular Disease

CYTOVAS Precision Cytomics

Maggie Schmierer[1], Wade Rogers[1], Todd Johnson[1], Kevin Judge[3], Noel Warner[3], Bob Cao[3], Joe Trotter[3], W. Roy Overton[1], Tatiana Arango[1], Michael McGrane[1], Eva Silvestro[1], Jonni Moore[2], Emile Mohler[2], Scott Damrauer[2]
[1]CytoVas, LLC, Philadelphia, PA, United States, [2]University of Pennsylvania, Philadelphia, PA, United States, [3]BD Biosciences, San Jose, CA, United States

BD

Penn University of Pennsylvania

Background

One of the fastest growing areas of flow cytometry research today is the exploration of the biological relevance of extracellular vesicles (EVs). In recent years, leaders in flow cytometry have proposed methods to minimize artifact in the measurement of EVs and to push instrumentation to maximum sensitivity. We have developed protocols for sample collection, transport, preparation and analysis of EVs. Samples were analyzed on a modified BD FACSCanto II 10-color cytometer (Canto Plus) fitted with a 200mW blue laser and a modified 200x400nm aperture in front of the SSC collection fiber to optimize small particle detection. (Figures 1a & 1b) A proprietary computational algorithm was developed to automate the analysis of EV data. These techniques were validated and have subsequently been utilized in multiple clinical research studies approved by the University of Pennsylvania IRB.

Methods

Blood collection: Fasting blood was drawn into a 2.7ml sodium citrate with 21G needle with 7-inch tubing.
Sample Transport: A gimbaled device was devised to keep tubes upright during transport to minimize platelet activation due to turbulence/shear.
Sample Preparation: Platelet Poor Plasma (PPP) was isolated within 6 hours using two 2500 x g, 15-minute centrifugation steps. PPP was then stained in a BD Trucount tube with an 8-color panel of vascular and inflammatory surface markers. (Table 1) All reagents were filtered with a 0.1um filter.
Data Acquisition: Serial dilution experiments were performed to determine plasma dilution factors that limit Poisson coincidence to negligible levels. (Figure 2) PPP stained PE threshold was determined equivalent to 144 MESF. (Figure 3) Mei Theory calculations confirm detection of EVs down to 106 nm. (Figure 4). Upper vesicle size cut-off was determined using a combination of 200 nm silica beads and platelet-rich plasma stained with CD41a. (Figure 4) Positivity thresholds were determined using a tritonized sample negative control. (Figure 5) Samples were collected using Side Scatter triggering with a window extension of 0.2.
Data Analysis: Fully automated proprietary data analysis algorithms were developed using R in order to eliminate subjectivity and to provide for exhaustive combinatorial analysis of all EV subsets. (Figure 6) These techniques were validated before the start of the study. (Figure 7)

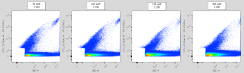
Results

An IRB-approved clinical study was conducted to test the ability of this assay to predict adverse cardiovascular events in 217 patients undergoing non-cardiac vascular surgery. A pre-surgery EV phenotype was discovered that distinguished patients experiencing a post-surgical adverse cardiac event ($P = 1.87 \times 10^{-5}$, after Bonferroni correction for multiple comparisons P = 0.002). (Figure 8)

Conclusions

A standardized platform provides the ability to reliably quantify vascular and inflammatory EVs in candidates for surgery, and perhaps in other disease populations where vascular damage is implicated such as autoimmune disorders, preeclampsia and HIV. Using these methods, we have developed a flow cytometry EV assay which may one day inform critical clinical decisions.

1a. Blue laser power optimization to 200mW for EV assay



1b. SSC gain and PE voltage titration

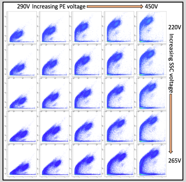
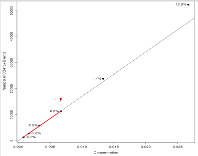


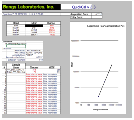
Table 1. 8-color vascular and inflammatory EV panel

Antibody (Ms-anti Hu)	Fluorochrome	BD Cat#
CD3- ASR	PerCp-Cy5.5	340948
CD105	AF647	561439
Annexin V	V500	561501
CD144	V450	561569
CD47	PE	556046
CD64	APC-R700	647701
CD31 (Dom1/2)	PE-Cy7	563651
CD41a	APC-H7	561422

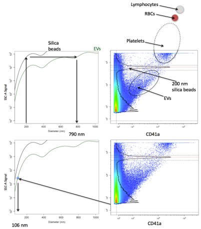
2. Serial dilution, minimizing Poisson coincidence



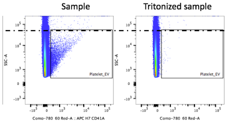
3. MESF calculation for PE threshold



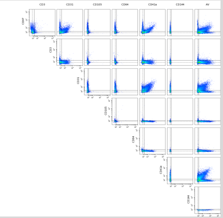
4. Mie theory visualization of EV detection to 106nm



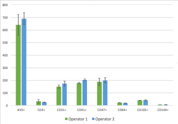
5. Triton negative control



6. Automated bi-variate sample analysis



7. EV Assay Variability: 2 Operators, 5 Sample Replicates



8. Pre-surgery EV phenotype distinguishes post-surgical cardiac event